Binding of Chara Myosin Globular Tail Domain to Phospholipid Vesicles

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Binding of Chara myosin globular tail domain to phospholipid vesicles was investigated quantitatively. It was found that the globular tail domain binds to vesicles made from acidic phospholipids but not to those made from neutral phospholipids. This binding was weakened at high KCl concentration, suggesting that the binding is electrostatic by nature. The dissociation constant for the binding of the globular tail domain to 20% phosphatidylserine vesicles (similar to endoplasmic reticulum in acidic phospholipid contents) at 150 mM KCl was 273 nM. The free energy change due to this binding calculated from the dissociation constant was $-37.3 \text{kJ mol}^{-1}$. Thus the bond between the globular tail domain and membrane phospholipids would not be broken when the motor domain of Chara myosin moves along the actin filament using the energy of ATP hydrolysis ($\Delta G^0 = -30.5 \text{kJ mol}^{-1}$). Our results suggested that direct binding of Chara myosin to the endoplasmic reticulum membrane through the globular tail domain could work satisfactorily in Chara cytoplasmic streaming. We also suggest a possible regulatory mechanism of cytoplasmic streaming including phosphorylation-dependent dissociation of the globular tail domain from the endoplasmic reticulum membrane.

Keywords: Ca$^{2+}$ regulation — Chara corallina — Cytoplasmic streaming — Globular tail domain — Plant myosin — Phospholipid binding.

Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; ER, endoplasmic reticulum; GST, glutathione S-transferase; GTD, globular tail domain; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIP$_2$, phosphatidylinositol 4,5-bisphosphate; PMSF, phenylmethylsulfonyl fluoride; PS, phosphatidylserine; TAME, N-$\beta$-tosyl-L-arginine methyl ester hydrochloride; TPCK, N-$\beta$-tosyl-L-phenylalanine chloromethylketone.

Introduction

Myosin is a molecular motor that moves along actin filaments using ATP as the energy source. Phylogenetic analysis of all the myosins sequenced so far revealed that there are at least 24 classes of myosin (Foth et al. 2006), though only three of them (classes VIII, XI and XIII) were found in plants (Knight and Kendrick-Jones 1993, Kinkema and Schiefelbein 1994, Vugrek et al. 2003). Several lines of evidence suggested that plant myosin XI plays important functional roles within cells in driving actin-based motility, such as intracellular vesicle transport and cytoplasmic streaming (Reddy 2001, Yamamoto 2007). Association with the organelle membrane is essential for its proper localization and function. However, little is known about the manner of binding of plant myosin XI to the membrane.

Detailed studies on this interaction with organelle membrane have been carried out on animal myosin V. It is known that each organelle has a specific receptor for the globular tail domain (GTD) of certain myosin V molecules, which allows it to be transported to its destination by the movement of the motor domain along actin filaments (Govindan et al. 1995, Hill et al. 1996, Catlett and Weisman 1998, Schott et al. 1999, Beach et al. 2000, Yin et al. 2000, Hoepfner et al. 2001, Boldogh et al. 2004, Itoh et al. 2004, Pushkova et al. 2006). Plant myosin XI has structural similarity to myosin V and has a similar GTD (Yamamoto et al. 1995, Kashiyma et al. 2000, Morimatsu et al. 2000, Tominaga et al. 2003). Hashimoto et al. (2005) reported that an antibody raised against a peptide with a sequence specific for Arabidopsis myosin XI (MYA2) co-localized with peroxisomes, suggesting specific interaction of MYA2 with this organelle. Reisen and Hanson (2007) recently expressed six Arabidopsis myosin XI tails fused with yellow fluorescent protein (YFP) in various plant cells and observed their association with unidentified vesicles that move at a velocity of about 1 $\mu$m s$^{-1}$.

On the other hand, it is known that animal myosin I can bind directly to the phospholipid bilayer through the interaction of its basic tail domain with the head group of acidic phospholipids such as phosphatidylinerine (PS; Adams and Pollard 1989, Hayden et al. 1990, Doberstein and Pollard 1992, Zot et al. 1992). It was shown that myosin XI from the freshwater alga Chara corallina also bound to PS vesicles (Yamamoto et al. 1995). This myosin is known to generate very fast cytoplasmic streaming in Chara cells (Yamamoto et al. 1994, Kashiyma et al. 2000, Ito et al. 2003). Hydrodynamic considerations suggested that
Chara myosin must move with bound organelles such as the endoplasmic reticulum (ER) to generate bulk water flow (Nothnagel and Webb 1982).

Specific association of myosin with a particular organelle is necessary for the transport of organelles to their destination. However, such an association with a specific organelle might not be necessary for Chara myosin if its function was only for cytoplasmic streaming. In this study, we expressed the GTD of Chara myosin fused with glutathione S-transferase (GST) and performed quantitative investigation of its binding to phospholipid vesicles to assess the possibility of its binding to organelles through phospholipids. We examined the GTD because it is the organelle-binding domain in most of myosins known so far (Kieke and Titus 2003).

Results

Globular tail domain construct
Chara myosin consists, from the N-terminus, of a motor domain having an ATPase site and an actin-binding site, a neck domain comprising six tandem repeats of IQ motifs (light chain-binding sites), an α-helical coiled-coil domain leading to dimer formation, and a globular tail domain (AB007459; Kashiyama et al. 2000). Analysis with COILS (Lupas 1996) revealed that the coiled-coil domain begins at Lys875 and ends at Val1,635. We assumed, therefore, that the GTD is from Val1,636 to Ala2,182. The cDNA region encoding Gly1,639–Ala2,182 of Chara myosin was fused with GST. The fused protein is composed of residues 1–221 of GST (all the amino acids residues of GST), a linker (EVLFQGPLGELEICSWYR) and residues 1,639–2,182 of Chara myosin. This fusion protein (calculated mol. wt. 89,392 Da) was expressed and purified as described in Materials and Methods.

Binding of Chara myosin GTD to phospholipid vesicles
It was reported that native Chara myosin co-sedimented with PS vesicles but not with phosphatidylcholine (PC) vesicles (Yamamoto et al. 1995). We first examined if Chara myosin interacted with PS vesicles through the GTD. A binding assay using the GTD fused with GST clearly showed that the GTD interacts with PS but not with PC (Fig. 1), suggesting that the phospholipid binding characteristics of Chara myosin are conferred by the GTD. Binding assays with other phospholipids revealed that the GTD interacts with acidic phospholipids such as PS and phosphatidylinositol (PI), but not with neutral phospholipids such as PC and phosphatidylethanolamine (PE) (Fig. 1). Since we used a fusion protein of GTD and GST, we tested if GST co-sedimented with these phospholipid vesicles. It was found that GST itself did not co-sediment with any of the phospholipids (data not shown).

Effect of ionic strength on the interaction between the GTD and PS vesicles
Because the charge of the phospholipid is an important factor for the interaction between the GTD and phospholipid vesicles, the electrostatic interaction is a candidate for the attractive force between them, as in the case of myosin I. To test this possibility, we examined the effect of KCl concentration on the interaction between the GTD (0.7 μM) and PS vesicles (300 μM). The amount of the GTD which co-sedimented with PS vesicles started to decrease at KCl concentrations >150 mM and only 10% of the GTD co-sedimented at 500 mM KCl (Fig. 2).

Binding affinity of GTD for PS vesicles
As mentioned before, myosin I binds to PS vesicles with very high affinity (Kd = 300 nM; Hayden et al. 1990). To estimate the binding affinity of the Chara myosin GTD to PS vesicles, we measured the dissociation constant for the binding of the GTD for PS vesicles under ionic conditions close to those in Chara cells [150 mM KCl, 2 mM MgCl2, 1 mM dithiothreitol (DTT), 0.5 mg ml⁻¹ bovine serum albumin (BSA) and 20 mM HEPES pH 7.4]. It was found that the dissociation constant was 72 ± 25 nM and the
maximum binding capacity was \((0.120 \pm 0.017) \times 10^{-1}\) mol GTD mol\(^{-1}\) PS (Fig. 3). The dissociation constant for \textit{Chara} myosin GTD was much smaller than that for myosin I even though the binding assay was done at a KCl concentration much higher than that used for myosin I (75 mM KCl, 2.5 mM MgCl\(_2\), 1 mM EGTA and 10 mM imidazole pH 7.5). Considering the effect of ionic strength on the interaction between GTD and PS vesicles as mentioned above (Fig. 2), the dissociation constant would be much smaller than 72 nM at 75 mM KCl.

**Affinity for mixed PS vesicles**

It is well known that the content of acidic phospholipids in natural organelle membranes is not 100%, and the acidic phospholipid content in bean cotyledon membrane vesicles is reported to be 20% (Allen et al. 1971). We therefore measured the binding of the GTD to vesicles made from 20% PS and 80% PC. The GTD concentration was varied from 0.05 to 10 µM at a mixed phospholipid concentration of 300 µM. We raised the concentration of mixed phospholipids to 300 µM to make the PS concentration the same as that used in the above-mentioned experiment (60 µM) and to make a measurable amount of the GTD co-sediment with the mixed phospholipid vesicles. Analysis of the amount of co-sedimented GTD showed that the dissociation constant for the binding to the mixed phospholipid vesicles was 273 ± 34 nM and the maximum binding capacity was \((0.930 \pm 0.079) \times 10^{-3}\) mol GTD mol\(^{-1}\) of the mixed phospholipids (Fig. 4).
Affinity for phosphatidyl inositol 4,5 bisphosphate

It is reported that one subclass of myosin I, myo1c, interacts specifically with phosphatidylinositol 4,5-bisphosphate (PIP2) and its binding to the plasma membrane is regulated by Ca ions and phospholipase C (Hokanson and Ostap 2006, Hokanson et al. 2006). We therefore studied the binding of the GTD (0.05–0.5 mM) to vesicles made from 2% PIP2 and 98% PC (total 300 mM). It was found that the dissociation constant was 157 ± 17 nM and the maximum binding capacity was (0.493 ± 0.013) × 10⁻³ mol GTD mol⁻¹ of the mixed phospholipid, respectively. Binding of mutant GTDs to PS vesicles

To investigate the site of electrostatic interaction, an arginine cluster of the GTD from Arg1,870 to Arg1,873 was altered to reduce its positive charge. Mutants RAAR and AAAA had the sequences Arg-Ala-Ala-Arg and Ala-Ala-Ala-Ala, respectively, instead of Arg-Arg-Arg-Arg in the wild-type GTD. The manner of binding of these mutant GTDs to PS vesicles was examined and compared with that of the wild-type GTD (Fig. 6). As seen in the figure, the binding affinity decreased drastically as the charge decreased, while the maximum binding capacity remained almost unchanged. The dissociation constants for the wild type, RAAR and AAAA were 72, 408 and 1,280 nM, respectively. The maximum binding capacities for the wild type, RAAR and AAAA were 0. 12 × 10⁻¹, 0. 12 × 10⁻¹ and 0. 14 × 10⁻¹ mol protein mol⁻¹ PS, respectively.

Discussion

Number of PS molecules interacting with the GTD

We showed in this study that the Chara myosin GTD binds strongly to acidic phospholipids through an electrostatic interaction (Figs. 1, 2). The affinity of the GTD for PS vesicles was much higher than that of myosin I whose direct interaction with plasma membrane phospholipid is well documented. The dissociation constant for the binding of brush border myosin I to PS vesicles was 300 nM and this value was obtained at 75 mM KCl. Our assay was done at a KCl concentration much closer to the physiological level (150 mM) and the dissociation constant was 72 nM (Fig. 3). The maximum binding capacity of PS vesicles for the GTD was 12 × 10⁻³ mol mol⁻¹ PS, and this value was of a similar order to that for brush border myosin I (5.5 × 10⁻³ mol mol⁻¹ PS). The value suggests that 1 mol of GTD occupies an area composed of about 40 molecules of PS if we consider the bilayer structure of the phospholipid vesicle membrane. This number is, of course, not the number of PS molecules interacting electrostatically with the GTD. The free energy change (ΔG°) due to the binding of the GTD to PS vesicles can be calculated from the binding constant K using the following equation:

ΔG° = -RT ln K
where $R$ and $T$ are the gas constant and absolute temperature, respectively. Since the binding constant is the inverse of the dissociation constant $K_d$, this equation can be rewritten as follows:

$$\Delta G^\ominus = RT \ln K_d$$

The free energy change due to the binding is, therefore, $\approx 40.6 \text{kJ mol}^{-1}$. The energy of electrostatic interaction in water is not as high as in a vacuum and is $<10 \text{kJ mol}^{-1}$ (Lodish et al. 2004). Therefore, the interaction of 4-5 positively charged amino acids with the head groups of PS would be sufficient for the binding of the GTD to PS vesicles. The maximum binding capacity of 2% PIP2 vesicles for the GTD is relevant to this question. Although one to one binding of the myosin tail to PIP2 was suggested (Hokanson and Ostap 2006), the Chara myosin GTD does not seem to be tethered by a single PIP2. If the binding of the GTD to PIP2 is one to one, the maximum binding capacity should be $0.02 \text{mol GTD mol}^{-1}$ of mixed phospholipid containing 2% PIP2. The value of the maximum binding capacity of $0.49 \times 10^{-3} \text{mol mol}^{-1}$ (Fig. 5) was much closer to $0.02 \times 0.02$, suggesting that at least two closely associated PIP2 molecules are required for the binding. Since the effective charge of PIP2 is $-4$ at neutral pH (McLaughlin et al. 2002), the number of molecules of PS (with a charge of $-1$) interacting with the GTD should be $>4$. The number of $>4$ is supported by the maximum binding capacity of 20% PS vesicles for the GTD (Fig. 4) because the value $0.930 \times 10^{-3} \text{mol mol}^{-1}$ is between $(0.2)^4$ and $(0.2)^5$.

The calculated area on the PS vesicle surface probably corresponds to a space physically occupied by the GTD, which would interfere with the binding of other GTD molecules. Our finding that the maximum binding capacity of PS vesicles for RAAR and AAAA mutants was almost the same as that for the wild type supports this idea. Since the center-to-center distance of phospholipid molecules in a bilayer is about 1 nm (Dufrene et al. 1997), the size of the space is about 40 nm$^2$. It is reported that the GTD of myosin V has a slightly elongated structure ($\sim 10 \times 4.5$ nm; Pashkova et al. 2006). The Chara myosin GTD together with GST also has an elongated structure and gyrates by Brownian motion around the binding site, which excludes the binding of other GTD molecules from the space of about 40 nm$^2$.

**The organelle to which Chara myosin GTD binds**

It is generally assumed that the organelle being pulled by Chara myosin is the ER because it exists most abundantly in cells and its well-developed meshwork structure is suitable for generating bulk flow of cytosol. Electron microscopy revealed that the tips of the ER membrane were attached to actin bundles in rapidly frozen cytoplasm of Chara (Kachar and Reese 1988). However, if the Chara myosin GTD binds to acidic phospholipids in the ER membrane, it should also bind to the vacuolar membrane and plasma membrane. Most of the Chara myosin molecules were localized around actin bundles when observed by immunofluorescence microscopy (Yamamoto et al. 2006) and their association with the vacuolar membrane or plasma membrane was scarcely observed (unpublished observation). The reason may simply be that the affinity for actin recruits the Chara myosin motor domain around the actin bundles while the GTD interacts with acidic phospholipids in the membrane of various organelles. The amount of myosin on the vacuolar membrane and plasma membrane will be small in the final equilibrium state because they are far from the actin bundles and because the head to tail length of the Chara myosin molecule is only $0.1 \mu$m (Yamamoto et al. 1995).

The Chara myosin GTD does not bind to chloroplasts, though they are the closest to the actin bundles, probably because the lipid composition of the outer membrane of their envelope is very different from that of other internal membranes. It is reported that the outer membrane of spinach chloroplast envelope contains large amounts of galactolipids (monogalactosyl diacylglycerol and digalactosyl diacylglycerol) and does not contain PE which exists commonly in the cytosolic leaflet of the internal and plasma membrane (Block et al. 1983). The polar lipid composition of the chloroplast envelope is similar to that of blue-green algae, except for PC (Murata et al. 1981), which probably reflects the evolutionary origin of chloroplasts.

The target of myosin generating cytoplasmic streaming in other plant cells may also be the ER because of the reasons mentioned above. However, the manner of binding to the ER membrane and the regulatory mechanism of cytoplasmic streaming may not be the same as in Chara. It is possible that other plant myosins bind to the ER membrane through receptors for the GTD because association of one subclass of myosin XI with a specific organelle (peroxisome) is reported in higher plants (Hashimoto et al. 2005).

As discussed above, the Chara myosin GTD can bind to acidic phospholipids in the membrane of any organelles, and it would bind many kinds of organelles when examined in vitro. It is the organization of actin filaments in Chara cells that makes the ER the most favorable organelle for the GTD to bind. We discuss in the following sections the binding between the GTD and ER membrane using data obtained from the binding of the GTD to 20% PS vesicles.

**Bond strength between GTD and the ER membrane**

The Chara myosin GTD could also bind strongly to 20% PS and 2% PIP$_2$ vesicles under conditions close to
Phospholipid binding of *Chara* myosin

Amount of *Chara* myosin molecules associated with the ER

Another important point to be considered is whether the amount of ER-bound *Chara* myosin is enough to maintain the flow of cytoplasm. Energy calculation suggested that only 1% of existing myosin molecules is sufficient to generate the force required for cytoplasmic streaming in *Chara* cells even if the efficiency of transducing chemical energy of ATP to mechanical force is as low as 10% (Yamamoto et al. 2006). The amount of ER-bound myosin can be calculated if we can estimate the amount of ER membrane in *Chara* cells or not because the binding force must be stronger than the force generated by the interaction between the motor domain of *Chara* myosin and actin. If this binding force was weak, myosin could not pull the ER to produce bulk water flow. Since the energy source for the motion of myosin is ATP, it would not exceed the energy liberated by ATP hydrolysis (30.5 kJ mol\(^{-1}\)). Our calculation suggests that the bond between the GTD and ER membrane in *Chara* cells is strong enough to allow the ER to be pulled by myosin for cytoplasmic streaming.

Sites of electrostatic interaction

Little is known about the phospholipid-binding sites on the surface of the *Chara* myosin GTD. Since charge reduction of the sequence from Arg1,870 to Arg1,873 drastically lowered its affinity for PS vesicles (Fig. 6), part of the binding site may be this arginine-rich sequence. However, there must be other binding sites because the AAAA mutant could still bind, though weakly, to PS vesicles (Fig. 6), and interaction with >4 PS molecules is suggested, as discussed above. There are other sequences rich in positively charged amino acids in the GTD (AB007459), e.g. Lys-Lys-Ser-Lys (from 1,765 to 1,768) and Arg-Leu-Lys-Lys (from 1,934 to 1,937). These sequences may also serve as binding sites for acidic phospholipids.

Regulatory mechanism of cytoplasmic streaming

If the membrane binding of the *Chara* myosin GTD is solely electrostatic in nature, phosphorylation of amino acid side chains near the basic amino acid cluster would weaken the binding, as in the case of RAAR and AAAA mutants (Fig. 6), and cause dissociation of moving myosin from the ER membrane. Since movement of myosin alone cannot generate bulk flow of water (Nothnagel and Webb 1982), such phosphorylation would stop cytoplasmic streaming in *Chara* cells. It is known that the cytoplasmic streaming stops when the intracellular Ca\(^{2+}\) concentration rises above...
10^{-6} M (Williamson and Ashley 1982, Kikuyama and Tazawa 1982). Tominaga et al. (1987) suggested that Ca^{2+} regulation involves a phosphorylation–dephosphorylation mechanism. In their scheme, streaming is inhibited when a protein component is phosphorylated by Ca^{2+}-dependent protein kinase, and streaming is activated when the same component is dephosphorylated. There are sequences around the arginine cluster of the Chara myosin GTD that match the consensus sequences of the Ca^{2+}-dependent protein kinase phosphorylation site (S/T X R/K) and the Ca^{2+}/calmodulin-dependent protein kinase II phosphorylation site (R X X S/T) (Fig. 7). Regulation of cytoplasmic streaming through the phosphorylation of the GTD by these kinases fits well with the scheme proposed by Tominaga et al. (1987), and a similar phosphorylation-dependent dissociation of the myosin V tail from organelles was reported (Karcher et al. 2001). Movement of Chara myosin along the actin filament without bound ER membrane may seem a waste of energy, but Chara myosin will dissociate and diffuse away from the actin filament immediately after one or two steps since it is a non-processive motor protein (Awata et al. 2001, Ito et al. 2007). Dissociated myosin molecules will rebind weakly to the ER membrane around them because there is no force to pull them apart from the ER membrane, or they may rebind strongly to the ER membrane after being dephosphorylated.

If phosphorylation of the GTD is a regulatory mechanism of cytoplasmic streaming in Chara cells, it will work efficiently when only myosin molecules moving along the actin filament are phosphorylated. Such selective phosphorylation will be possible if kinase molecules are tethered near actin filament bundles. McCurdy and Harmon (1992) studied the localization of Ca^{2+}- dependent protein kinase in Chara cells by immunofluorescence microscopy and showed its localization on actin filament bundles and on small organelles associated with the bundles.

We, of course, noticed that this regulatory mechanism is one of many possible mechanisms. Although the motor activity of Chara myosin is known not to be inactivated directly by Ca^{2+} (Awata et al. 2001), it can be inactivated by Ca^{2+}-dependent phosphorylation of the motor domain. Phosphorylation of the motor domain may allow it to interact with the GTD, and this interaction may dissociate the GTD from the ER. We previously reported that the affinity of Chara myosin for PS vesicles becomes high (more myosin molecules co-sedimented) in the presence of Ca^{2+} (Yamamoto et al. 1995). However, we did not observe any effect of Ca^{2+} on the affinity of the GTD for PS vesicles when we used sucrose-loaded vesicles (data not shown). Since divalent cations are known to catalyze lipid aggregation and fusion (Wilschut et al. 1985), our previous report on the effect of Ca^{2+} may have been due to changes in vesicle size and structure. Sucrose-loaded vesicles sedimented equally regardless of Ca^{2+}, but a larger amount of unloaded vesicles probably sedimented with bound myosin in the presence of Ca^{2+} in our previous study. A similar observation was reported for myosin I by Hokanson and Ostap (2006).

Materials and Methods

Reagents
1-α-phosphatidylserine (PS), 1-α-phosphatidyethanolamine (PE) and PIP2 were purchased from Avanti Polar Lipids (Alabaster, AL, USA). 1-α-phosphatidylinositol (PI) was obtained from Sigma (St Louis, MO, USA). Nα-p-tosyl-1-arginine methyl ester hydrochloride (TAME), Nα-p-tosyl-l-phenylalanine chloromethylketone (TPCK), leupeptin, pepstatin A and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma. Glutathione–Sepharose® 4B is a product of Amersham Biosciences (Buckinghamshire, UK). Malachite green oxidate was purchased from Chroma-Gesellschaft (Germany). SYPRO-red was obtained from Molecular Probes Inc. (Eugene, OR, USA).

Expression and purification of the GTD of Chara myosin
The fusion gene of GST and the GTD was subcloned into the baculovirus transfer vector pFastBac1 (Invitrogen, Carlsbad, CA, USA) between the Xhol and XhoI sites. This plasmid (pFastBac1-GST-GTD) was introduced into Escherichia coli DH10Bac cells to produce bacmid DNA. Purified bacmid DNA encoding GST–GTD was used to infect Sf-9 cells to produce baculovirus containing the GST–GTD gene. The GST–GTD fusion protein was expressed in High Five® cells and purified as follows. Cells were harvested and washed with 150 mM NaCl and 10 mM Tris–HCl pH 7.4. Collected cells were sonicated in a lysis buffer containing 200 mM NaCl, 1 mM EDTA, 0.1 mM EGTA, 30 mM Tris–HCl pH 7.4 and protease inhibitor mixture (50 μg/ml TAME, 40 μg/ml TPCK, 5 μg/ml leupeptin, 3 μg/ml pepstatin A and 0.1 mM PMSF), and intact cells were removed by centrifugation at 30,000 g for 30 min. Supernatant was centrifuged further at 260,000 g for 30 min and the resultant supernatant was mixed with glutathione–Sepharose® 4B resin equilibrated with the lysis buffer and left for 4 h with gentle agitation. The resin was packed in a column and washed first with a 40-fold column volume of the lysis buffer and then with a solution containing 150 mM KC1 and 10 mM Tris–HCl pH 7.4. The resin was suspended with one column volume of glutathione.

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\text{C}^2+\text{ dependent protein kinase phosphorylation site}
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\text{C}^2+/\text{calmodulin dependent protein kinase phosphorylation site}
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Fig. 7 Phosphorylation sites near the arginine cluster. Filled triangles indicate threonine residues that can be phosphorylated by Ca^{2+}-dependent protein kinase (S/T x R/K). Open triangles indicate serine or threonine residues that can be phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II (R x x S/T).

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\text{C}^2+\text{ dependent protein kinase phosphorylation site}
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solution (150 mM KCl, 20 mM glutathione, 100 mM HEPES pH 8.0), left for 30 min with gentle agitation, and the released protein was collected. This procedure was repeated five times. Glycerol was added to this protein solution to make 10% for preservation.

**Mutant GTDs with altered electrostatic charge**

The wild-type GTD has the sequence Arg-Arg-Arg-Arg from 1,870 to 1,873 (AB007459). Since this basic arginine cluster is a candidate for the acidic phospholipid-binding site on the GTD as shown in the Results, we altered this sequence to Arg-Ala-Ala-Ala or Ala-Ala-Ala-Ala by PCR-based site-directed mutagenesis (ExSite mutagenesis kit, Stratagene, La Jolla, CA, USA). The primers used to generate the Arg-Ala-Ala-Ala mutation were 5'-CGGCCCAAGAACGGTGTCCGGG-3' and 5'-CGCGGCGTGAGTTGACCCACC-3'. For the Ala-Ala-Ala-Ala-Ala mutation were 5'-CGCCAAGAACGGTGTCCGGG-3' and 5'-CGGTGCTGGAGTTGACCCACC-3'. After verifying their sequences, the mutant genes were subcloned into pFastBac1-GST-GTD and then expressed and purified as mentioned above for the wild-type GST–GTD.

**Preparation of phospholipid vesicles**

Phospholipid vesicles were prepared according to Hayden et al. (1990) with slight modification. Phospholipids dissolved in chloroform–methanol were dried in vacuo and suspended in a solution containing 176 mM sucrose and 20 mM HEPES pH 7.4 by sonication. The large aggregate was removed by centrifugation and the homogenous supernatant was dialyzed against 150 mM KCl and 20 mM HEPES pH 7.4. This sucrose-loaded phospholipid vesicle suspension was used within 3 d of preparation.

**Measurement of phospholipid concentration**

The concentration of phospholipid was determined by measuring acid-labile phosphate. We placed the phospholipid vesicle suspension (100 μl) into a test tube and evaporated the solvent by heating with a gas burner. The test tube was cooled and 1 ml of 9.24 M perchloric acid was added to it. Then the test tube was sealed with a screw cap and heated at 150°C for 6 h. The solution was diluted 30-fold with glass-distilled water. An aliquot was withdrawn and mixed with an equal volume of a malachite green solution containing 0.7 N HCl, 0.03% malachite green oxalate, 0.2% sodium molybdate and 0.05% Triton X-100. This mixture was kept at 30°C for 25 min and the absorption at 650 nm was measured. The phosphate concentration was determined from the standard curve made by measuring the color development of a known amount of phosphate.

**Binding assay**

Binding of the Chara myosin GTD to phospholipid vesicles was determined by incubating vesicles made from various phospholipids with the GTD (0.05–1 μM) in the presence of 80–500 mM KCl, 2 mM MgCl$_2$, 0.5 mM DTT, 10 μg ml$^{-1}$ leupeptin, 0.7 μg ml$^{-1}$ pepstatin A, 0.5 mg ml$^{-1}$ BSA, 10 mM Tris–HCl pH 7.4 at 25°C for 20 min, followed by centrifugation at 95,000 × g for 30 min at 25°C to separate the vesicle-bound GTD from the unbound form. BSA was included to prevent non-specific adsorption of the GTD to the centrifuge tubes. Both the supernatant and pellet were recovered and treated with SDS sample buffer. Protein samples were resolved by SDS-PAGE and gels were stained either with Coomassie brilliant blue or with SYPRO-red. Gels stained with SYPRO-red were scanned by using a Molecular Imager FX (Bio-Rad, Hercules, CA, USA), and data were analyzed by Quantity One (Bio-Rad).

**Acknowledgments**

This work was supported by a Grant-in-Aid for Scientific Research and Grant-in-Aid for Scientific Research on Priority Areas from The Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Phospholipid binding of Chara myosin


(Received July 20, 2007; Accepted September 26, 2007)